Prostaglandin F2 promotes embryo implantation and development in the pig

Kaczyński, Piotr ; Baryla, Monika ; Goryszewska, Ewelina ; Bauersachs, Stefan ; WacŁawik, Agnieszka

Abstract: Successful establishment and development of pregnancy requires proper communication between developing conceptuses and the maternal reproductive tract. Prostaglandins are key players involved in the regulation of reproductive processes in mammals including pigs. Due to its luteolytic action, prostaglandin F2-alpha (PGF2) is mainly considered as an undesirable factor during early pregnancy. However, its content in the uterine lumen is elevated in pigs and other mammals. Recently, we reported an important role of PGF2 in the endometrium during early pregnancy in the pig. Thus, the aim of the present study was to determine whether PGF2 can act on porcine trophoblast and if so, to elucidate what effect it could exert. We detected increased expression of PGF2 receptor during the implantation period (from day 14 until day 19 of pregnancy). Global gene expression profiling using microarrays and quantitative PCR studies revealed that PGF2 acting on porcine trophoblast cells in vitro alters expression of genes potentially involved in processes related to implantation, such as: cell proliferation, focal adhesion, extracellular matrix binding, cell migration, cytoskeleton organization, immune interactions, ion homeostasis, and lipid metabolism. Using primary porcine trophoblast cells, we demonstrated that PGF2 stimulated trophoblast cell proliferation and adhesion to extracellular matrix protein. This was likely mediated by mitogen-activated protein kinases (MAPK1/3) and focal adhesion kinase (FAK) since we observed increased phosphorylation of MAPK1/3 and FAK in trophoblast cells treated with PGF2 . To conclude, the present report indicates a novel role for PGF2 in the porcine conceptus as a para- and autocrine factor supporting pregnancy establishment.

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1 **TITLE**

2 Prostaglandin F2α promotes embryo implantation and development in the pig

3 **AUTHORS**

4 Piotr Kaczynski[1], Monika Baryla[1], Ewelina Goryszewska[1], Stefan Bauersachs[2], Agnieszka
5 Waclawik[1]

6 [1] Institute of Animal Reproduction and Food Research, Polish Academy of Sciences,
7 Tuwima 10, 10-748 Olsztyn, Poland.

8 [2] University of Zurich, Vetsuisse Faculty, Clinic of Reproductive Medicine, Genetics and
9 Functional Genomics, Eschikon 27 EHB 23.1, 8315 Lindau, Switzerland

10 **SHORT TITLE**

11 New role of prostaglandin F2α in trophoblast

12 **CORRESPONDING AUTHOR AND PERSON TO WHOM REPRINT REQUEST**
13 **SHOULD BE ADDRESSED:**
14 Agnieszka Waclawik Ph.D.,
15 Institute of Animal Reproduction and Food Research of Polish Academy of Sciences,
16 Tuwima 10, 10-748 Olsztyn, Poland
17 Phone: +48 89 539 31 11, +48 89 535 74 22
18 Fax: +48 89 535 74 21
19 e-mail: waclawik@pan.olsztyn.pl

ABSTRACT

Successful establishment and development of pregnancy requires proper communication between developing conceptuses and the maternal reproductive tract. Prostaglandins are key players involved in the regulation of reproductive processes in mammals including pigs. Due to its luteolytic action, prostaglandin F2-alpha (PGF2α) is mainly considered as an undesirable factor during early pregnancy. However, its content in the uterine lumen is elevated in pigs and other mammals. Recently, we reported an important role of PGF2α in the endometrium during early pregnancy in the pig. Thus, the aim of the present study was to determine whether PGF2α can act on porcine trophoblast and if so, to elucidate what effect it could exert. We detected increased expression of PGF2α receptor during the implantation period (from day 14 until day 19 of pregnancy). Global gene expression profiling using microarrays and quantitative PCR studies revealed that PGF2α acting on porcine trophoblast cells in vitro alters expression of genes potentially involved in processes related to implantation, such as: cell proliferation, focal adhesion, extracellular matrix binding, cell migration, cytoskeleton organization, immune interactions, ion homeostasis, and lipid metabolism. Using primary porcine trophoblast cells, we demonstrated that PGF2α stimulated trophoblast cell proliferation and adhesion to extracellular matrix protein. This was likely mediated by mitogen-activated protein kinases (MAPK1/3) and focal adhesion kinase (FAK) since we observed increased phosphorylation of MAPK1/3 and FAK in trophoblast cells treated with PGF2α. To conclude, the present report indicates a novel role for PGF2α in the porcine conceptus as a para- and autocrine factor supporting pregnancy establishment.

Introduction

The establishment and development of pregnancy in the pig, as in any mammalian species, requires proper communication between developing conceptuses and the intrauterine environment. This embryo-maternal dialog includes hormonal and immune interactions precisely controlled by hormones, cytokines, growth factors and other molecules interacting in endo-, para- and autocrine manners.

During the maternal recognition of pregnancy period, which occurs in pigs on days 11-12 of pregnancy, conceptuses secrete increased levels of estrogens that are one of the major conceptus signals for the establishment of pregnancy. This leads to the redirection of luteolytic prostaglandin F2α (PGF2α) secretion from endocrine to exocrine (to the uterine lumen) mode, and also affects the hormonal environment in the reproductive tract, resulting in prolonged lifespan of the corpus luteum (CL) and, thereby, maintained progesterone (P4) synthesis (Bazer & Thatcher 1977). The second phase of increased estrogen secretion occurs in pigs on day 15 of pregnancy and lasts until day 25 after fertilization (Geisert *et al.* 1990).

Key factors involved in the control of reproductive processes in mammals are prostaglandins (PGs). Their importance for pregnancy establishment and development is shown by the fact that lack of PG synthesis leads to termination of pregnancy just before the implantation process in mammals such as pigs (Kraeling *et al.* 1985), cattle (Erdem & Guzeloglu 2010) and rodents (Kennedy *et al.* 2007). Interestingly, intrauterine administration of prostaglandin synthase inhibitors resulted in improper conceptus elongation in sheep (Dorniak *et al.* 2011) which has a much different programming for conceptus growth compared to rapid trophoblast elongation in

the pig. In contrast, PG synthesis inhibition does not affect elongation of porcine conceptuses (Geisert *et al.* 1986) but it does affect their survival during the attachment period (Kraeling *et al.* 1985) Prostaglandin E2 (PGE2) and PGF2α are the major prostaglandins involved in reproductive functions in mammalian reproductive tracts. They act through their receptors: PTGER1-4 (PGE2 receptors isoforms 1-4) and PTGFR (PGF2α receptor). The expression profiles of PTGER2 and PTGER4 were determined in porcine trophoblasts at different stages of development (Waclawik *et al.* 2013). However, the expression profile of PTGFR in porcine conceptuses during early pregnancy has not been studied yet. Interestingly, the expression of PGE2 and PGF2α synthases is elevated both in porcine endometrium and in conceptuses during the maternal recognition of pregnancy and/or implantation (Waclawik *et al.* 2006, Waclawik & Ziecik 2007). PGE2 has been described as playing a luteoprotective/luteotrophic role (Waclawik *et al.* 2009, Waclawik *et al.* 2008, Waclawik 2011) whereas PGF2α has been mainly perceived as a luteolytic factor in sheep, cattle and pigs, as well as in rats, rabbit and hamsters (reviewed in McCracken *et al.* 1999). Thus, it could have also a detrimental effect in pregnancy development in mammals, including human, cattle, pigs and dogs (reviewed in Kaczynski et al. 2016). However, it should not be regarded as undesirable factor in pregnancy because elevated amounts of PGF2α in the uterine lumen and/or its increased endometrial synthesis occur not only in pigs (Waclawik *et al.* 2006, Zavy *et al.* 1980) but also in other mammals including rodents, cattle, dogs and humans (Kennedy *et al.* 2007, Ulbrich *et al.* 2009, Wang *et al.* 2010, Kowalewski *et al.* 2014, Vilella *et al.* 2013). Moreover, a novel role for PGF2α in the porcine endometrium during the peri-implantation period has been recently reported by our group, indicating that PGF2α participates in pregnancy establishment by promoting angiogenesis and expression of genes

involved in endometrial remodeling and embryo-maternal communication (Kaczynski *et al.* 2016).

Previously we indicated that synthesis of PGF2α during the implantation period is elevated both in the porcine endometrium and the conceptus (Waclawik *et al.* 2006, Waclawik & Ziecik 2007) Recently we also elucidated the mechanism of PGF2α action in the endometrium (Kaczynski & Waclawik 2013; Kaczynski *et al.* 2016). Hence, the question arose whether PGF2α can act through its receptor in the conceptus and if so, what could be the role of PGF2α in porcine trophoblast cells? During the period when the endometrium becomes receptive for embryo implantation, both the uterine luminal epithelium and conceptus cells simultaneously initiate the adhesion cascade (Geisert *et al.* 2015). Attachment of trophoblast cells to luminal epithelium is supported by interactions between integrins and extracellular matrix (ECM) proteins. Integrin receptors (AVB1, AVB3, AVB5 and A4B1) are able to bind to the RGD (Arg-Gly-Asp) sequence present in osteopontin, fibronectin and vitronectin proteins (Bowen *et al.* 1996, Johnson *et al.* 2003). Our studies indicate the important role of PGE2 in activation of intracellular pathways in porcine trophoblast cells related to adhesion (mitogen activated protein kinases 1/3 - MAPK1/3; focal adhesion kinase (FAK) and intracellular adhesion molecule 1 (ICAM1) and promotion of trophoblast adhesion to extracellular matrix (ECM) proteins (Waclawik *et al.* 2013). Moreover, we demonstrated a stimulatory effect of PGE2 on trophoblast cell estradiol-17β synthesis (Waclawik *et al.* 2013). It has been shown that lack of PGs (PGE2 and PGF2α) decreased adhesion of mouse blastocysts and JEG-3 spheroids to human luminal epithelial cells (Vilella *et al.* 2013). However, the mechanisms by which PGF2α may stimulate trophoblast cell adhesion to ECM proteins remain unknown. Furthermore, despite elevated

conceptus and endometrial synthesis of PGF2α, its role in the proliferative capacities of trophoblast cells and activation of intracellular pathways is still unresolved.

Based on data in the literature including results from our previous studies, we hypothesized that during early pregnancy in the pig, PGF2α acting through its receptor in trophoblast cells activates intracellular pathways important for the implantation process. Thus, the objectives of the present study were: (i) to determine the expression profile of the PTGFR gene and protein in porcine conceptuses/trophoblasts at different stages of development; (ii) to investigate the effect of PGF2α on global gene expression profiles in porcine primary trophoblast cells in vitro; (iii) to study the effect of PGF2α on trophoblast cell adhesion and proliferation; and (iv) to evaluate whether PGF2α may activate MAPK1/3 and FAK in porcine trophoblast cells, which could be involved in adhesion and/or proliferation processes .

MATERIALS AND METHODS

All procedures involving animals were conducted in accordance with the national guidelines for agricultural animal care and were approved by the Animal Ethics Committee, University of Warmia and Mazury in Olsztyn, Poland, permission No. 36/2012.

Experiment 1: PTGFR mRNA and protein expression in the porcine conceptus/trophoblast.

Gilts after two natural estrous cycles were bred at the onset of estrus (day 0) and then 12 h and 24 h later. Pregnant gilts were slaughtered at the local abattoir. Conceptuses at the pre-implantation stage were flushed from the uterine horns with 20 mL of sterile phosphate-buffered saline (PBS; 137 mM NaCl, 27 mM KCl, 10 mM Na2HPO4 and 2 mM KH2PO4, pH 7.4). Conceptuses/trophoblasts at the implantation and early placentation stage were dissected from the endometrium. Considering the days of pregnancy and morphology of trophoblasts, the

conceptus samples derived from the same animal were pooled and classified into the following groups: days 10-12 (preimplantation stage; spherical and tubular forms, n=5), days 14-15 (beginning of attachment/implantation; filamentous forms, n=5), days 16-19 (filamentous forms; implantation stage, n=7) and days 20-25 (chorion which originates from trophoblast; the onset of placenta development; n=10). Dissections of trophoblast tissues from conceptuses was performed since day 18 post mating (Waclawik *et al.* 2013). Therefore, expression of PTGFR was analyzed in 10- to17-day conceptuses and 18- to 25-day trophoblast tissues. Collected conceptuses/trophoblasts were snap-frozen in liquid nitrogen and stored at -80 °C until gene and protein expression analyses.

RNA isolation and cDNA synthesis

Collected tissues were homogenized in 800 μL of Fenozol buffer (A&A Biotechnology, Gdansk, Poland) using Lysing Matrix D beads (MP Biomedicals, Santa Ana, USA) and Fast Prep homogenizer (MP Biomedicals). RNA was isolated from tissues with RNA Mini Kit (A&A Biotechnology), according to the manufacturer's protocol. Integrity and concentration of isolated RNA was analyzed by using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). Additionally, concentration of RNA was measured with NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA). To generate cDNA for qPCR reaction total RNA sample (1 μg) was reverse-transcribed with MultiScribe™ Reverse Transcriptase kit (Thermo Fisher Scientific) according to the manufacturer's protocol. cDNA samples were stored in -80 °C for further quantitative PCR (qPCR) analyses.

Quantitative PCR

Analysis of *PTGFR* gene expression in conceptuses was performed as described previously (Kaczynski & Waclawik 2013). Reverse-transcribed cDNA (3.5 μL) was added to the reaction mixture: 12.5 μl Power SYBR Green master mix (Thermo Fisher Scientific), 2.5 μl of each sense and antisense primer (1 μM; Supplementary Table 1), and 4 μl of nuclease-free water. Specific primers used for real-time RT-PCR are listed in Table 1. The PCR program for *PTGFR* gene was performed as follows: initial denaturation (95 °C, 10 min) followed by 36 cycles of denaturation (95 °C; 15 sec), annealing and elongation (60 °C; 1 min). For actin beta (*ACTB*), cyclophilin A (*PPIA*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) amplification the PCR program was: initial denaturation (95 °C, 10 min) followed by 36 cycles of denaturation (95 °C, 15 sec), annealing (55 °C, 30 sec) and elongation (72 °C 1 min). After PCR, melting curves were acquired by gradual increases in the temperature from 60 to 95 °C to ensure that a single product was amplified in the PCR reaction. All real-time PCR reactions were performed with Applied Biosystems 7900HT Real-Time PCR system (Life Technologies, Carlsbad, USA). Gene expression was estimated using real-time PCR Miner software (Zhao & Fernald 2005). Stability of the reference genes in the porcine conceptuses was assessed using the statistical algorithms Normfinder 2.0 (Andersen *et al.* 2004). Three reference genes were analyzed: *GAPDH*, *PPIA* and *ACTB*. The most stable combination of reference genes was the geometrical mean of *ACTB* and *GAPDH* expression values.

Protein isolation

Conceptuses (days 10-17)/trophoblasts (days 18-25) collected from pregnant gilts were homogenized on ice in 150 μL of homogenization buffer (50 mM Tris–HCl, pH 8.0; 150 mM NaCl, 1 mM EDTA) supplemented with protease inhibitor cocktail (Sigma–Aldrich, Saint Louis, USA). Homogenates were then centrifuged for 15 min at 800 × g at 8 °C and stored at – 80 °C

for further analysis. The protein concentration was determined with Bradford Assay. PTGFR protein abundance in conceptus homogenates was assessed by Western Blot analysis.

Western Blot

Western Blot analysis for PTGFR protein expression in conceptuses was performed as described previously (Kaczynski & Waclawik 2013). Briefly, equal amounts of protein samples (40 µg) were separated on 10% SDS PAGE. Proteins were electroblotted onto 0.2 mm PVDF membrane. After blocking in 5% nonfat dry milk, the blots were incubated overnight at 4 °C with primary antibodies (Table 2) and afterwards with 1:20000 dilution of secondary anti-rabbit horseradish peroxidase antibodies (Bio-Rad Laboratories, Hercules, USA) for 90 min at room temperature. Immune complexes were visualized using Clarity Western ECL Substrate (Bio-Rad). Sample loading was standardized to expression of GAPDH. Signal was quantitated using Versa-Doc 4000M imaging system (Bio-Rad Laboratories).

Experiment 2: The effect of PGF2α on global gene expression profile in porcine trophoblast cells

Incubation of the porcine primary trophoblast cells with PGF2α.

Porcine trophoblast cells were isolated as described previously (Waclawik *et al.* 2013). Crossbred gilts (n=5) of similar age and genetic background, were inseminated and slaughtered on day 15 of pregnancy at the local abattoir. Conceptuses were recovered from uteri by gentle flushing of each uterine horn with medium M199 (Sigma-Aldrich) containing 0.1% (wt/vol) bovine serum albumin (BSA), penicillin (100 IU/mL, Sigma-Aldrich), and streptomycin (100 µg/mL, Sigma-Aldrich), warmed up to 37°C. Conceptuses were separated from flushing medium by centrifugation for 10 min at 150 × g at 8°C. Trophoblast tissue was dissected from the

197 embryonic disc region. Trophoblast cells were then non-enzymatically dispersed by pipetting.
198 After dispersion, the cells were washed three times with sterile medium M199 with antibiotics
199 and 0.1% BSA, resuspended in sterile M199 containing antibiotics and 5% newborn calf serum
200 (NCS, Sigma-Aldrich) and seeded on human collagen I-coated 6-well plates (BioCoat™ 356400;
201 BD Biosciences, Bedford, USA). Adherent cells were washed with PBS 48 h after plating to
202 remove contaminating aggregates. Additionally, to assess the purity of cell cultures, isolated
203 cells were stained for the presence of the trophoblast marker - cytokeratin as described
204 previously (Blitek et al., 2012). After reaching 70-80% confluence, the cells were washed with
205 sterile PBS. The following treatments were performed: PGF2α (100 nM; 1 μM; Sigma-Aldrich)
206 and control (medium M199 with vehicle). Treated cells were incubated for 24 h at 37 °C in
207 humidified atmosphere containing 95% air and 5% CO2. After incubation, culture media were
208 collected and frozen. Cells were lysed with Fenozol buffer (A&A Biotechnology), harvested and
209 stored at -80°C until isolation of RNA.

210 *Global gene expression profiling by using expression microarrays*

211 Total RNA was isolated from trophoblast cell lysates as described in experiment 1 and destined
212 for downstream analyses: global gene expression profiling (expression microarray analysis) and
213 qPCR. Microarray analysis was performed starting from 100 ng total RNA isolated from
214 trophoblast cells after *in vitro* experiment (Experiment 2). Cy3-labeled cRNA was produced with
215 the Low-Input Quick Amp Labeling Kit, one-color (Agilent Technologies), and hybridized to the
216 Agilent 4x44k Porcine Gene Expression microarrays (G2519F-026440) according the
217 manufacturer's instructions. Hybridized and washed slides were scanned at 2-μm resolution with
218 an Agilent DNA Microarray Scanner (G2505C; Agilent Technologies). Image processing was
219 performed with Feature Extraction Software 10.5.1.1 (Agilent Technologies). Signals were

filtered based on "well above background" flags (detection in three of four samples) and normalized with the BioConductor package VSN (Huber *et al.* 2002). For quality control, normalized data were analyzed with a distance matrix and a heatmap based on pair-wise distances (BioConductor package Geneplotter). Differentially expressed genes (DEGs) were identified in the contrast (pairwise comparison) "PGF2α-treated cells" vs. "vehicle-treated cells" using the BioConductor package LIMMA (Linear Model for Microarray Analysis) (Smyth 2004). Low number of genes showing expression differences leads to a too strong correction of nominal P-values and a very low power to detect true DEGs (Hackstadt & Hess 2009). Regarding the sensitivity problem of the FDR in case of a low number of DEGs, the cut-off values were set as follows: ≥1.2-fold difference in expression values with a p-value less than 0.05. Significant probes were annotated based on mapping of the probe sequences (60 nt sequences) to the porcine genome (SusScrofa 11.1). Known and potential human ortholog or homolog genes were assigned using a custom ortholog annotation database (Mammalian Ortholog and Annotation Database, MOAdb; JT Bick, SE Ulbrich and S Bauersachs, unpublished data). If a gene in the list was represented by more than one probe sequence, the mean fold-change and p-value were calculated. To visualize the distribution of expression signals of DEGs among samples, hierarchical clustering using Pearson correlation distance was performed using Multiexperiment Viewer software (MeV) (Saeed *et al.* 2003).

Functional annotation of microarray data

Lists of DEGs generated by LIMMA analysis were used as input data for functional annotation. To study the effect of PGF2α on the global gene expression profile in trophoblast cells, open-source and commercial software was applied. The Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang *et al.* 2009a, Huang *et al.*2009b) was used to calculate

the fold enrichment of identified gene ontology (GO) terms using following databases: Functional Categories (UP_KEYWORDS); gene ontology Biological Process (GOTERM_BP_FAT), Cellular Component (GOTERM_CC_FAT), Molecular Function (GOTERM_MF_FAT); General Annotations (SP_COMMENT); Pathways (BIOCARTA; KEGG PATHWAY); Protein Domains (INTERPRO) and Protein Interactions (UCSC_TFBS). Results generated by DAVID were summarized in tabular format. Topp Cluster software (Kaimal *et al.* 2010) was used to identify gene ontologies enriched by DEGs and to generate a network showing the shared and list-specific functional features. Results were summarized in tabular format and visualized by interaction network. To identify signaling pathways, molecular networks, and biological functions for DEGs Ingenuity Pathway Analysis (IPA; v. 01.12; Qiagen, Redwood City, USA) were used. Results were summarized in tabular and in graphic formats.

Validation of microarray results

To verify results obtained in microarray analysis we validated the expression of *ADAM9*, *AGER*, *AMBP*, *BMP2*, *DECR1*, *IL1A*, *KNG1*, *LPAR1*, *MASP1*, *MFGE8*, *MMP2*, *PROX1*, *S100G*, *SLCO2A1*, *STC2*, *THBS1*, *TLR3* and *VTN* genes using real-time RT-PCR method. Additionally, based on our previous studies (Kaczynski *et al.* 2016) and literature data we studied whether PGF2α affected the expression of *BGN*, *IL6*, *IL6R*, *MMP9*, *TGFB3*, *TIMP1* and *TIMP2* genes. Based on microarray results, we also decided to evaluate the gene expression profile of *MMP9* in porcine conceptuses (days 10-17)/ trophoblasts (days 18-25) throughout pregnancy development. cDNA was generated from RNA isolated from trophoblast cells as described in Experiment 1. Real-time RT-PCR was performed using TaqMan assays (Thermo Fisher Scientific; Table 1) accordingly to the manufacturer's protocol. Results from qPCR were analyzed as described in

Experiment 1. Stability of the reference genes in the porcine trophoblast cells was assessed using the statistical algorithm Normfinder 2.0 (Andersen *et al*. 2004). Three reference genes were analyzed: *GAPDH*, *PPIA* and *ACTB*. The most stable combination of reference genes was the geometrical mean of *ACTB* and *GAPDH* expression values.

Experiment 3: The effect of PGF2α on trophoblast cell adhesion and proliferation.

Porcine primary trophoblast cells were isolated as described previously (Waclawik *et al.* 2013) with some modifications. Briefly, recovered conceptuses were washed three times with medium M199 supplemented with 5% (v/v) NCS and 0.1% (m/v) BSA. Subsequently, trophoblast tissue underwent mild digestion in 0.25% trypsin solution (Biomed, Lublin, Poland) for 30 min at 37°C. After digestion, solution was neutralized with M199 supplemented with 10% (v/v) NCS. Cells were then centrifuged at 150 × g for 10 min and washed three times with medium M199 supplemented with 5% (v/v) NCS. After washing the cells were re-suspended in medium M199 with 1% (v/v) NCS. Viability and cell number was assessed with trypan blue dying.

For adhesion assay, cells (1×10^6 cells/mL) were pre-incubated with medium M199 containing vehicle (0.01% ethanol) or PGF2α (100 nM, 1 μM) with/without 50 μM of PTGFR antagonist (AL8810, Sigma Aldrich) for 30 min at 37°C. Subsequently, 100 μL of each cell suspension was transferred onto Millicoat Cell Adhesion Strips coated with human fibronectin (ECM101; Millipore, Billerica, USA) and incubated for 3 h at 37°C. All treatments were performed in triplicates. After incubation, cell adhesion was measured using colorimetric method accordingly to the manufacturer's protocol.

Cells used in proliferation assay were re-suspended in culture medium (M199 supplemented with 5% NCS) in concentration 5×10^4 cells/mL. After attachment, the cells were incubated for 48 h

288 at 37°C in humidified atmosphere (95% air, 5% CO_2) with medium M199 containing vehicle
289 (0.01% ethanol) or PGF2α (100 nM, 1 μM) or stable PGF2α analogue (fluprostenol; 100 nM, 1μ
290 M) with/without 50 μM of PTGFR antagonist (AL8810). Based on our previous results
291 (Kaczynski *et al*. 2016), 20% NCS was used as a positive control. After 48 h of treatment,
292 proliferation was determined using the Cell Titer 96 Aqueous One Solution Proliferation Reagent
293 (Promega) according to the manufacturer's instructions. Experiments were repeated 6 times in
294 triplicates. Fold difference was determined by dividing the absorbance obtained by PGF2α-
295 treated cells (with/without inhibitors) by the absorbance obtained by vehicle-treated cells.

296 **Experiment 4:** Effect of PGF2α on downstream targets potentially involved in porcine
297 trophoblast cells adhesion and proliferation.

298 To determine the protein expression of downstream targets of PGF2α involved in porcine
299 trophoblast cells adhesion and proliferation, we used previously described (Waclawik *et al.*
300 2013) *in vitro* model in which primary trophoblast cells were incubated for 24 h with either
301 vehicle (0.01% ethanol) or PGF2α (100 nM, 1 μM) in the presence/absence of 50 μM AL8810.
302 After incubation cells were lysed with RIPA buffer (PBS; pH 7.4; 1% Triton X-100; 0.5%
303 sodium deoxycholate; 0.1% sodium dodecyl sulfate; 1 mM EDTA) containing Protease Inhibitor
304 Cocktail (Sigma-Aldrich) and Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich). Content of
305 MAPK1/3, pMAPK1/3, FAK and pFAK in cell homogenates was determined using Western blot
306 and was normalized against GAPDH expression. Phosphorylation of MAPK and FAK protein
307 was calculated by dividing the relative content of phosphorylated to non-phosphorylated protein.

308 *Western Blot*

MAPK1/3 and FAK protein phosphorylation was evaluated using Western Blot analysis as described previously (Waclawik *et al.* 2013). Briefly, equal amounts of protein samples (18 µg) from conceptus cell lysates were separated on 10% SDS PAGE. Proteins were electroblotted onto 0.2 mm PVDF membrane. After blocking in 5% bovine serum albumin blots were incubated overnight at 4 °C with primary antibodies (Table 2) and afterwards with 1 : 20 000 dilution of secondary anti-rabbit horseradish peroxidase antibodies (Bio-Rad Laboratories) for 90 min at room temperature. Immune complexes were visualized using Clarity Western ECL Substrate (Bio-Rad). Sample loading was standardized to expression of GAPDH. Signal was quantitated using Versa-Doc 4000M imaging system (Bio-Rad Laboratories).

Statistical analyses

Results from *in vitro* experiments (cell adhesion and proliferation assays), Western Blot and qPCR studies were analyzed by one-way ANOVA followed by Tukey's test. All statistical analyses were conducted using GraphPad PRISM v. 6.01 software (GraphPad Software Inc., San Diego, USA).

RESULTS

Experiment 1: Expression of PTGFR gene and protein increases in the conceptus/trophoblast during the implantation period.

Expression of PTGFR mRNA was elevated in conceptuses/trophoblasts from day 14 until day 19 of pregnancy (the implantation period; $p<0.05$) compared with conceptuses/trophoblasts on days 10-12 (the preimplantation period). The highest expression of PTGFR mRNA was observed in conceptuses/trophoblasts from days 16-19 of pregnancy (Fig. 1A) and was 48-fold greater than in conceptuses/trophoblasts from days 10-12 of pregnancy ($p<0.05$). PTGFR protein expression

was elevated at the implantation period (days 14-15, 16-19 of pregnancy) when compared to later stage (days 20-25 of pregnancy; $p<0.05$; Fig. 1B). Expression of PTGFR protein in day 10-12 conceptuses was not evaluated due to insufficient amount of material for both gene and protein analyses. Thus, in collected material only expression of PTGFR gene was possible to analyze.

Experiment 2: Effect of PGF2α on global gene expression profile in porcine trophoblast cells.

Characterization of trophoblast cells

The purity of the isolated cells was assessed by the evaluation of the presence of the trophoblast marker - cytokeratin and cell morphology. Isolated trophoblast cells presented uninuclear morphology (Suppl. Fig. 1A). Positive immunoreaction for cytokeratin confirmed the epithelial phenotype of trophoblast cells (Suppl. Fig. 1B). Antiserum-specific isotype control (negative control) used at the same dilution and protein concentration as the primary antibody is shown in Supplemental Figure 1B (lower-left panel). Purity of cell cultures was assessed at 98%.

Global gene expression profiling by using expression microarrays

The porcine trophoblast cells' response towards the PGF2α treatment was characterized using a porcine Agilent microarray assay. Differential gene expression between the two experimental groups ("PGF2α-treated cells" vs. "vehicle-treated cells") was evaluated. The number of detected probes passing the filters was 27045. Pairwise distance analysis based on all detectable probes revealed grouping replicates, but differences between PGF2α-treated and vehicle-treated samples are also indicated in the heat map (Suppl. Fig. 2). Using LIMMA package, 810 probes were found as differentially regulated (fold change $< / > 1.2$; $p<0.05$; Suppl. Table 1). Annotation of probe sequences revealed 576 DEGs (216 down-regulated, 360 up-regulated) affected by PGF2α.

The five DEGs showing highest upregulation in the pairwise comparison "PGF2α-treated cells" vs. "vehicle-treated cells" were obtained for the following genes: *S100G* (3.9-fold), *NPEPPS* (2.6-fold), *PDE1B* (2.5-fold), *KNG1* (2.3-fold), *AMBP* (2.0-fold), whereas the five most downregulated genes (suppressed by PGF2α) were: *SELENBP1* (-1.8-fold), *DIO1* (-1.8-fold), *ENTPD8* (-1.8-fold), *LYZ* (-1.7-fold), *FMO1* (-1.6-fold). Hierarchical clustering of the obtained DEGs based on Pearson correlation revealed treatment grouping of analyzed samples with two main distinct clusters of genes with uniform distribution of expression signals (Fig. 2).

Functional annotation of microarray data

Functional terms enriched for up- and down-regulated DEGs from both experimental groups were identified by DAVID analysis. For up-regulated DEGs, DAVID identified 83 functional annotation clusters with enrichment scores from 1.04 up to 3.96 (Suppl. Table 2), whereas for down-regulated DEGs 62 functional annotation clusters were identified with enrichment scores from 1.06 up to 6.79 (Suppl. Table 3). The most interesting functional terms of overrepresented annotation clusters enriched for up-regulated genes were related to focal adhesion, cell migration, hypoxia, response to fibroblast growth factor (FGF2) and transforming growth factor beta (TGFB), and regulation of cell proliferation and MAPK cascade (Table 3). Terms overrepresented by down-regulated genes were mainly related to fatty acid biosynthetic process, cell migration, anion transport, response to interferon gamma, and placenta development (Table 3). Complete results from Functional Annotation Clustering were summarized in Supplementary Tables 2 and 3.

Using ToppCluster software, GO terms (biological process, cellular component and molecular function) and pathways (KEGG) enriched by DEGs were identified and summarized in tabular format (Suppl. Table 4). Similarly, as in DAVID analysis, overrepresented terms were related to

processes potentially important for embryo implantation and development of early pregnancy such as: adhesion, immune response, placenta development, morphogenesis, cytoskeleton organization and others. The most interesting enriched terms from PGF2α-up-regulated and PGF2α-down-regulated clusters were visualized as a network (Fig. 3).

Using Ingenuity Pathway Analysis on 577 DEGs, the most interesting over-represented canonical pathways were associated with regulation of lipid metabolism, inflammation, and cholesterol catabolism (LXR/RXR; p=3.47E-06), as well as with integrin (p=2.2E-04) and mTOR (p=1.701E-02) signaling (Suppl. Table 5). The main functional terms derived from IPA analysis showed similar results, compared to DAVID analysis, with highly involved functional terms such as embryonic development, inflammatory response, cellular growth and proliferation and lipid metabolism. (Suppl. Fig. 3; Suppl. Table 6). Upstream analysis revealed that identified DEGs can be also regulated by other factors than PGF2α factors (Suppl. Table 7). The most interesting factors potentially stimulating DEGs were estradiol-17β, HOXA10 and TGFB1 and 2.

Effect of PGF2α on the selected gene expression in porcine conceptuses.

Validation of microarray results using qPCR confirmed expression of 14 out of 18 selected genes (*IL1A*, *MFGE8*, *MMP2*, *SLCO2A1*, *STC2*, *ADAM9*, *AGER*, *AMBP*, *BMP2*, *LPAR1*, *MASP1*, *S100G*, *THBS1* and *VTN*), whereas 4 of them (*KNG1*, *PROX1*, *DECR1* and *TLR3*) showed diverse expression in the qPCR to that detected by microarray analysis (Suppl. Table. 8). Results from qPCR revealed that PGF2α (100 nM) acting on trophoblast cells stimulated expression of *ADAM9*, *VTN*, *LPAR1*, *BMP2*, *DECR1* and *THBS1* genes ($p<0.05$). Treatment of the trophoblast cells with 1 μM of PGF2α resulted in increased expression of *AGER*, *AMBP*, *BGN*, *IL6R*, *S100G*, *TLR3* and *MASP1* genes ($p<0.05$) and in down-regulation of *MFGE8*, *SLCO2A1*, *TIMP1*, *TIMP2*, *KNG1*, *MMP9*, *SCT2* and *TGFB3* gene expression ($p<0.05$). Expression of *IL1A*, *MMP2*

and *PROX1* genes was decreased in the trophoblast cells treated with both doses (100 nM; 1 uM) of PGF2α ($p<0.05$). Results from qPCR analyses are shown in the Figure 4. Additionally, we evaluated the expression profile of *MMP9* in conceptuses (days 10-17) / trophoblasts (days 18-25) at different stages of development. We found that the abundance of *MMP9* mRNA was lowest at the period corresponding to maternal recognition of pregnancy (days 10-12 of pregnancy) and at the beginning of implantation (days 14-15). Expression of *MMP9* was intermediate during implantation (days 16-19 of pregnancy) and markedly increased during placenta development (days 20-25 of pregnancy; $p<0.05$; Fig. 4E).

Experiment 3: PGF2α stimulates adhesion and proliferation of the porcine trophoblast cells.

We demonstrated that PGF2α (100 nM; 1 μM) acting through its receptor increased adhesion of trophoblast cells to fibronectin ($p<0.05$; Fig. 5A). Stimulating effect of PGF2α (100 nM and 1 μM) was blocked by using PTGFR antagonist (AL8810). Al8810 used alone did not have any effect on cell adhesion.

During early pregnancy in mammals, rapid transformation of conceptus morphology and conceptus cell differentiation should be accompanied by increased cell proliferation. In the present study, we evaluated the influence of PGF2α on conceptus cell proliferation during implantation period. We found that PGF2α (100 nM) as well as its stable analog – fluprostenol (100 nM), acting through PTGFR stimulated proliferation of porcine trophoblast cells ($p<0.05$; Fig. 5B). The effects of PGF2α (100 nM) and fluprostenol (100 nM) were abolished by using AL8810. Al8810 used alone had effect similar to the control.

Experiment 4: PGF2α acting on trophoblast cells increases phosphorylation of factors potentially involved in adhesion and proliferation processes.

Since we previously indicated involvement of FAK and MAPK (Waclawik *et al.* 2013) in adhesion of trophoblast cells and as MAPK signaling is also involved in cell proliferation we determined whether PGF2α may stimulate phosphorylation of these proteins in the trophoblast cells. In the present study, we demonstrated for the first time that PGF2α stimulated phosphorylation of MAPK1/3 (Fig. 5C) and FAK (Fig. 5D) proteins ($p<0.05$) in porcine trophoblast cells and that these effects were mediated through PGF2α receptor (Fig. 5C and D).

DISCUSSION

In the present study, we demonstrated for the first time the para- and/or autocrine mechanisms of prostaglandin F2α action on conceptus/trophoblast cells during the implantation period. We also described the expression profile of PGF2α receptor (PTGFR) in porcine conceptuses at different stages of their development.

Results from our experiments discussed below provide a new perspective on the role of PGF2α in embryo-maternal communication in the pig. We postulate that PGF2α, which is highly abundant in the uterine lumen during early pregnancy, is not an undesirable factor but instead is an important hormone supporting processes related to proper embryo development and implantation. In the present study, we examined, for the first time, the expression profiles of PTGFR mRNA and protein in porcine conceptuses/trophoblasts during early pregnancy. Maximal expression of the PTGFR gene in conceptuses/trophoblasts occurred during the implantation period. Similarly, increased abundance of PTGFR protein was observed on days 14-19 of pregnancy. These results concur with our previous findings that PGF2α synthase protein expression is increased in conceptuses/trophoblasts from days 14 to 25 compared to days 10-12 of pregnancy (Waclawik & Ziecik 2007). Strong signals of PTGFR protein expression are observed in the ovine trophectoderm on days 16, 18 and 20 of pregnancy (Dorniak *et al.* 2011).

444 PGs secreted together with IFNT by ovine conceptuses regulate endometrial gene expression and
445 functions that are important for conceptus growth and development (Dorniak *et al.* 2012). Our
446 results are also consistent with transcriptomic data, in which expression of the PTGFR gene in
447 day 14 filamentous conceptuses was 14-fold greater than in day 10 (spherical) -12 (filamentous)
448 conceptuses (Ross *et al.* 2009). Interestingly, we indicated the up-regulation of endometrial
449 PTGFR protein during the implantation period (Kaczynski & Waclawik 2013). These results
450 strongly support the hypothesis that PGF2α secreted by the endometrium and conceptuses into
451 the uterine lumen can act on trophoblasts in an auto- and/or paracrine manner.

452 In the present study, we evaluated the effect of PGF2α on global gene expression profiles in
453 porcine conceptuses at the onset of the implantation period. Using different bioinformatics
454 software, we were able to demonstrate that PGF2α potentially affects a broad range of processes
455 and functions related mainly with focal adhesion, extracellular matrix binding, cytoskeleton
456 organization, cell migration and proliferation, but also with immune processes, metabolism,
457 response to hypoxia, growth factor signaling and ion homeostasis. Intriguingly, similar processes
458 (e.g., protein kinase regulator activity, cell motility, transport) identified in conceptuses
459 undergoing the transition from day 12 to day 14 filamentous forms (Ross *et al.* 2009) were also
460 observed in trophoblast cells treated with PGF2α. Moreover, it is interesting that analysis of
461 potential up-stream regulators of genes which were differentially regulated by PGF2α revealed
462 that these DEGs may be also regulated by other factors such as estradiol-17β, HOXA10 or TGFB
463 which are important players in the embryo-maternal dialogue during early pregnancy (reviewed
464 in Waclawik *et al.* 2017).

465 Due to the relatively low fold change threshold for detection of DEGs it is recommended to
466 validate these results for corresponding DEGs if more in-depth studies are projected based on our

transcriptomic analyses results. In the present study, we validated part of the microarray results using qPCR and functional in vitro experiments. Validation of expression of particular genes of interest (*AGER, AMBP, MASP1, MFGE8, PROX1, SLCO2A1, VTN, ADAM9, LPAR1, BMP2, DECR1, THBS, S100G, STC2, IL1A* and *TLR3*) confirmed overall the results from the microarray analyses. Additionally, we analyzed the PGF2α-mediated expression of genes that are potentially important in embryo-maternal interactions during the implantation period (*BGN, MMP2, MMP9, TIMP1, TIMP2, KNG1, TGFB3, IL6, IL6R* and *TGFB3*). Results of the microarray and qPCR analyses revealed that the PGF2α affected the expression of genes related to processes crucial for successful implantation, such as: ECM organization, ECM protein binding, focal adhesion, inflammatory response, cell proliferation, migration and motility or activation of intracellular protein kinases. Here, we briefly discuss the importance of validated genes in the most important processes accompanying the implantation process.

In all mammalian species, during the peri-implantation period formation of the placenta is preceded by adhesion of trophoblast cells to luminal epithelium of the endometrium (Geisert *et al.* 2015). This requires a profound rearrangement of endometrial tissue that is essential for successful implantation of embryos. Reorganization of tissue structures is related to processes such as ECM remodeling and vasculature development and is tightly controlled by a number of factors. Metalloproteinases are factors regulating ECM remodeling. MMPs together with their associated inhibitors (TIMPs) act concomitantly regulating aspects of reproductive function (reviewed in Curry & Osteen 2003). MMP2 and MMP9 were reported to mediate human trophoblast cell invasion into matrigel *in vitro* (Bischof *et al.* 1995). Recently, we reported PGF2α-stimulated expression of the MMP9 gene in porcine endometrium, suggesting its involvement in ECM remodeling (Kaczynski *et al.* 2016). In this study, we observed a decrease

490 in *MMP2* and *MMP9* mRNA content in porcine trophoblast cells in response to PGF2α
491 treatment. Simultaneously, we noticed that PGF2α up-regulated expression of the *TIMP2* gene
492 which is an inhibitor of MMP2 and MMP9. These results correspond to the *MMP9* gene
493 expression profile in porcine conceptuses at different stages of development that was evaluated
494 in the present study. Intriguingly, we found that PGF2α acting on human trophoblast cells has an
495 opposite effect, i.e. it increases *MMP9* gene expression that is probably related to invasive type
496 of implantation of human trophoblast (Baryla *et al.* 2017). We suggest that the down-regulation
497 of *MMP2* and *9* and up-regulation of *TIMP2* mRNA expression in porcine conceptuses could be
498 an effect of action of endometrium which by secreting PGF2α may protect the endometrial tissue
499 against excessive degradation of its ECM and this may be related to the non-invasive type of
500 implantation specific for the pig. Moreover, we demonstrated that PGF2α differentially
501 regulated other genes (*BGN, THBS, VTN, MFGE8, ADAM9, KNG1, AMBP* and *PROX1)* that
502 were reported to be involved in ECM remodeling, cell adhesion and proliferation in different
503 mammalian species (Kaczynski *et al.* 2016, Bowen *et al.* 1996, Couchman *et al.* 1990,
504 Kolakowska *et al.* 2017, Bloor *et al.* 2002, Taylor *et al.* 1997, Barua *et al.* 2017, Kim *et al.* 2006,
505 Vonnahme *et al.* 2004, Hettinger *et al.* 2001, Kim *et al.* 2013). Results from functional *in vitro*
506 experiments performed herein confirmed results from gene expression studies and support the
507 conclusion that PGF2α acting through its receptor in trophoblast cells stimulates adhesion of
508 trophoblast cells to ECM proteins. Interestingly, we recently demonstrated that the role of PGE2
509 in increasing trophoblast adhesion is not species-specific because the mechanism is PTGER2-
510 and integrin-dependent in both porcine and human trophoblast cells (Waclawik *et al.* 2013).
511 Moreover, iloprost (prostacyclin analogue) acting through prostacyclin membrane receptors
512 (PTGIR) stimulated porcine day 14 trophoblast cells to adhere to fibronectin (Morawska-

513 Pucinska *et al.* 2014). Thus, the present report emphasizes the role of prostaglandins in proper
514 control of the adhesion cascade during the implantation period in the pig.

515 During the peri-implantation period, porcine conceptuses undergo rapid transformation. On day
516 12 of pregnancy, trophoblasts elongate to more than 100 millimeters in length (Anderson, 1978,
517 Geisert *et al.* 1982). It has been stated that the conceptus elongation process is more dependent
518 on cell outgrowth than cell division (Perry, 1981). However, elongation is not the only process
519 occurring during conceptus development. Cellular differentiation is also very important for
520 embryonic development in all mammalian species. This process requires tight control over cell
521 proliferation, migration and cell-cell interaction. The potential role of TGFB, LPA1, PROX1,
522 AMBP and BMP2 in pregnancy establishment was reported in a number of mammals (Kim *et al.*
523 2013, Blitek *et al.* 2013, Jeong *et al.* 2016, McKeehan *et al.* 1986, Lee *et al.* 2007). Interestingly,
524 IL6 which is mostly related to immunological processes has been reported to stimulate
525 trophoblast cell proliferation and promote their adhesion to fibronectin (Blitek *et al.* 2012). In the
526 present study, we found that PGF2α did not affect *IL6* mRNA, however, it increased IL6
527 receptor (*IL6R*) mRNA content in porcine day 15 trophoblast cells. The present study is the first
528 demonstrating the role of PGF2α in promoting trophoblast cell proliferation in any mammalian
529 species. We recently reported an indirect influence of PGF2α on uterine endothelial cell
530 proliferation (Kaczynski *et al.* 2016). Moreover, fluprostenol (PGF2α analogue) was reported to
531 stimulate the expression of proteins related to proliferation of bovine endometrial cells (Zhang *et*
532 *al.* 2017).

533 During the implantation period in some mammals, including pigs both pro- and antiinflammatory
534 processes are activated at the embryo-maternal interface and the proper balance between these
535 two kinds of immunological responses is crucial for successful development of pregnancy

(reviewed in Waclawik 2011). In the present study, we found that PGF2α differentially regulated the expression of *KNG1, MFGE8, AMBP, IL1, IL6R, MASP1, AGER* and *TLR3* genes that are involved in modulation of immunological processes in many species (Vonnahme *et al.* 1999, Allen *et al.* 2002, Tan *et al.* 2015, Tuo *et al.* 1996, Prins *et al.* 2012, Wang *et al.* 2007, Bebraum *et al.* 2008, Zhang *et al.* 2007). Thus, we infer that PGF2α by differentially regulating genes in trophoblast cells may contribute to the control of immune interactions between developing conceptuses and the endometrium.

The importance and function of calcium during early embryo development and implantation have been well studied (reviewed in Whitaker 2006). Binding endometrial epithelial cells to trophoblast cells results in calcium influx in endometrial epithelial cells in humans (Tinel *et al.* 2000). In the porcine uterine lumen, concentration of calcium ions increases during the peri-implantation period (Geisert *et al.* 1982). Bikunin, one of the products of the *AMBP* gene, has been reported to inhibit the release of intracellular calcium ions induced by lipopolysaccharide (Kanayama *et al.* 1995). Stanniocalcin (STC), a glycoprotein regulating calcium homeostasis and has been suggested to be a luminal epithelial marker for implantation in pigs (Song *et al.* 2009). Another factor related to calcium ion homeostasis in endometrium during pregnancy is S100G protein also known as calbindin-d9k (Choi *et al.* 2009). Our data indicate that PGF2α by regulating *AMBP*, *STC2* and *S100G* gene expression in porcine conceptuses may be an upstream regulator of factors involved in calcium homeostasis. Our data suggest that in porcine conceptuses PGF2α regulates *MFGE8* and *DECR1* which are involved in fatty acid metabolism, fatty-acid resorption and placental transport (Khalifeh-Soltani *et al*, 2013; Kunau *et al*, 1978, Marques *et al*, 2009).

Prostaglandin signaling is crucial for proper conceptus development (Dorniak *et al.* 2011, Kennedy *et al.* 2007). SLCO2A1 was suggested to play a critical role in supporting the establishment and maintenance of pregnancy by regulating PG transport at the maternal–fetal interface in pigs. Expression of *SLCO2A1* mRNA in day 15 conceptuses is decreased compared to day 12 conceptuses (Seo *et al.* 2014). Accordingly, we found down-regulation of *SLCO2A1* gene expression in porcine trophoblast cells in response to PGF2α treatment. This may suggest that PGF2α by regulating its own transporter expression in trophoblast cells may autoregulate its secretion by conceptuses during implantation period.

Processes accompanying the implantation process such as cell adhesion and proliferation are triggered by activation of particular intracellular signaling pathways. Results from gene expression studies indicated that PGF2α enriched terms related to activation of protein kinases, such as MAP kinase activity and protein phosphorylation. In the present study, we demonstrated that PGF2α stimulated MAPK1/3 and focal adhesion kinase (FAK) protein phosphorylation in trophoblast cells. Interestingly, PGF2α has been reported to stimulate tyrosine phosphorylation of p125 FAK in NIH-3T3 cells (Watanabe *et al.* 1994) and in 293-EBNA cells stably expressing isoform A of the PTGFR prostanoid receptor (Pierce *et al.* 1999). PTGFR is a G-protein-coupled receptor activating Gq11 protein (Bos *et al.* 2004) and it has been demonstrated that genes up-regulated at the end of gestation in a Gαq/11-dependent manner in ovarian (granulosa/luteal) cells included genes involved in focal adhesion and extracellular matrix interactions (Waite *et al.* 2016). Moreover, in human adenocarcinoma cells (Ishikawa cell line), PGF2α was able to induce MAPK1/3 phosphorylation (Sales *et al.* 2005). We also found that PGE2 induced phosphorylation of MAPK1/3 and FAK. Taking all the above facts together, we speculate that PGF2α acting through its receptor in trophoblast cells may activate Gq11 protein that leads to

increased MAPK1/3 and FAK phosphorylation. This may result in enhanced adhesion of porcine trophoblast cells to ECM proteins.

In summary, the present study indicates a new role for PGF2α secreted both by the conceptuses and endometrium during the implantation period in pigs. Results from global gene expression profiling indicate that PGF2α acting in an auto- and/or paracrine manner is involved in processes important for conceptus development and attachment to the uterine epithelium (cell proliferation and focal adhesion, extracellular matrix binding, cell migration, cytoskeleton organization, immune interactions, ion homeostasis, metabolism, activation of TGFB and FGF signaling pathways, response to hypoxia). Functional *in vitro* experiments confirmed insights from transcriptomic studies, allowing us to conclude that PGF2α by inducing transcriptomic changes in porcine trophoblast cells affects a broad range of processes potentially involved in implantation and development of the placenta, particularly activation of intracellular protein kinases (MAPK, FAK) and stimulation of trophoblast cell adhesion and proliferation.

DECLARATION OF INTERESTS

Authors of the manuscript declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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608 **REFERENCES**

609 **Allen MR, Zhang BR, Hettinger AM, Goad DW, Malayer JR & Geisert RD** 2002 Detection
610 of bradykinin and bradykinin-b2 receptors in the porcine endometrium during the estrous cycle
611 and early pregnancy. *Biology of Reproduction* **66**:574–579.

612 **Andersen CL, Jensen JL & Ørntoft TF** 2004 Normalization of real-time quantitative reverse
613 transcription-PCR data: a model-based variance estimation approach to identify genes suited for
614 normalization, applied to bladder and colon cancer data sets. *Cancer Research* **64**(15):5245-
615 5250.

616 **Anderson LL** 1978 Growth, protein content and distribution of early pig embryos. *Anatomical*
617 *Record* **190**:143–153

618 **Barua S, Macedo A, Kolb DS, Wynne-Edwards KE & Klein C** 2017 Milk-fat globule
619 epidermal growth factor 8 (MFGE8) is expressed at the embryo– and fetal–maternal interface in
620 equine pregnancy. *Reproduction Fertility and Development* **30**:585-590.

621 **Baryla M, Kaczynski P & Waclawik A** 2017 Prostaglandin F2α regulates adhesion of
622 HTR8/SVneo trophoblast cell line. *Fertility 2017 Conference Materials* p 64.

623 **Bazer FW & Thatcher WW** 1977 Theory of maternal recognition of pregnancy in swine based
624 on estrogen controlled endocrine versus exocrine secretion of prostaglandin F2α by the uterine
625 endometrium. *Prostaglandins* **14** 397–400.

626 **Berbaum K, Shanmugam K, Stuchbury G, Wiede F, Körner H & Münch G** 2008 Induction
627 of novel cytokines and chemokines by advanced glycation end products determined with a
628 cytometric bead array. *Cytokine* **41**:198–203.

Bischof P, Martelli M, Campana A, Itoh Y, Ogata Y & Nagase H 1995 Importance of matrix metalloproteinases in human trophoblast invasion. *Early Pregnancy* 1(4):263-239.

Blitek A, Morawska-Pucinska E, Szymanska M, Kiewisz J & Waclawik A 2013 Effect of conceptus on transforming growth factor (TGF) β1 mRNA expression and protein concentration in the porcine endometrium--in vivo and in vitro studies. *Journal of Reproduction and Development* **59**(6):512-519.

Blitek A, Morawska E & Ziecik AJ 2012 Regulation of expression and role of leukemia inhibitory factor and interleukin-6 in the uterus of early pregnant pigs. *Theriogenology* **78**(5):951-964.

Bloor DJ, Metcalfe AD, Rutherford A, Brison DR & Kimber SJ 2002 Expression of cell adhesion molecules during human preimplantation embryo development. *Molecular Human Reproduction* **8**(3):237-245.

Bos CL, Richel DJ, Ritsema T, Peppelenbosch MP & Versteeg H 2004 Prostanoids and prostanoid receptors in signal transduction. *International Journal of Biochemistry and Cell Biology* **36**(7):1187-1205.

Bowen JA, Bazer FW & Burghardt RC 1996 Spatial and temporal analyses of integrin and Muc-1 expression in porcine uterine epithelium and trophectoderm in vivo. *Biology of Reproduction* **55**:1098–1106.

Choi Y, Seo H, Kim M & Ka H 2009 Dynamic expression of calcium-regulatory molecules, TRPV6 and S100G, in the uterine endometrium during pregnancy in pigs. *Biology of Reproduction* **81**(6):1122-1130.

650 **Couchman JR, Austria MR & Woods A** 1990 Fibronectin-cell interactions. *Journal of*
651 *Investigative Dermatology* **94**(6 Suppl):7S-14S.

652 **Curry TE Jr & Osteen KG** 2003 The matrix metalloproteinase system: changes, regulation,
653 and impact throughout the ovarian and uterine reproductive cycle. *Endocrine Reviews* **24**(4):428-
654 465.

655 **Dorniak P, Bazer FW & Spencer TE** 2011 Prostaglandins regulate conceptus elongation and
656 mediate effects of interferon tau on the ovine uterine endometrium. Biology of Reproduction **84**
657 1119–1127.

658 **Dorniak P, Bazer FW, Wu G & Spencer TE** 2012 Conceptus-derived prostaglandins regulate
659 endometrial function in sheep. *Biology of Reproduction* **9**:1-7.

660 **Erdem H & Guzeloglu A** 2010 Effect of meloxicam treatment during early pregnancy in
661 Holstein heifers. *Reproduciton in Domestic Animals* **45**:625–628.

662 **Geisert RD, Rasby RD, Minton JE, Wetteman RP 1986** Role of prostaglandins in
663 development of porcine blastocysts. *Prostaglandins* **31:191-203.**

664 **Geisert RD, Zavy MT, Moffatt RJ, Blair RM & Yellin T** 1990 Embryonic steroids and the
665 establishment of pregnancy in pigs. *Journal of Reproduction and Fertility Supplement* **40**:293–
666 305.

667 **Geisert AD, Johnson GA & Burghardt RC** 2015 Implantation and Establishment of Pregnancy
668 in the Pig. In: Geisert RD, Bazer FW (eds.) Regulation of Implantation and Establishment of
669 Pregnancy in Mammals. *Advances in Anatomy Embryology and Cell Biology* Springer
670 International Publishing, Switzerland 137-182.

671 **Geisert RD, Renegar RH, Thatcher WW, Roberts RM & Bazer FW** 1982 Establishment of
672 pregnancy in the pig: I. Interrelationships between preimplantation development of the pig
673 blastocyst and uterine endometrial secretions. *Biology of Reproduction* **27**:925–939.

674 **Hackstadt AJ, Hess AM.** 2009 Filtering for increased power for microarray data analysis. *BMC*
675 *Bioinformatics*; 10:11.

676 **Hettinger AM, Allen MR, Zhang BR, Goad DW, Malayer JR & Geisert RD** 2001 Presence
677 of the acute phase protein, bikunin, in the endometrium of gilts during estrous cycle and early
678 pregnancy. *Biology of Reproduction* **65**(2):507-513.

679 **Huang DW, Sherman BT & Lempicki RA** 2009a Bioinformatics enrichment tools: paths
680 toward the comprehensive functional analysis of large gene lists. Nucleic Acids Research
681 **37**(1):1-13.

682 **Huang DW, Sherman BT & Lempicki RA**. 2009b Systematic and integrative analysis of large
683 gene lists using DAVID Bioinformatics Resources. *Nature Protocols* **4**(1):44-57.

684 **Huber W, von Heydebreck A, Sultmann H, Poustka A & Vingron M** 2002 Variance
685 stabilization applied to microarray data calibration and to the quantification of differential
686 expression. *Bioinformatics* **18**(Suppl 1):S96–S104.

687 **Jeong W, Seo H, Sung Y, Ka H, Song G & Kim J** 2016 Lysophosphatidic Acid (LPA)
688 Receptor 3-Mediated LPA Signal Transduction Pathways: A Possible Relationship with Early
689 Development of Peri-Implantation Porcine Conceptus. *Biology of Reproduction* **104**:1-11.

690 **Johnson GA, Burghardt RC, Bazer FW & Spencer TE** 2003 Minireview: osteopontin: roles
691 in implantation and placentation. *Biology of Reproduction* **69**:1458–1471.

Kaczynski P & Waclawik A 2013 Effect of conceptus on expression of prostaglandin F2α receptor in the porcine endometrium. *Theriogenology* **79**:784-790.

Kaczyński P, Kowalewski MP & Waclawik A 2016 Prostaglandin F2α promotes angiogenesis and embryo-maternal interactions during implantation. *Reproduction* **151**:539-552.

Kaimal V, Bardes EE, Tabar SC, Jegga AG & Aronow BJ 2010 ToppCluster: a multiple gene list feature analyzer for comparative enrichment clustering and network-based dissection of biological systems. *Nucleic Acids Research* **38** (Web Server issue):W96-102.

Kanayama N, Halim A, Maehara K, Kajiwara Y, Fujie M & Terao T 1995 Kunitz-type trypsin inhibitor prevents LPS-induced increase of cytosolic free Ca2+ in human neutrophils and HUVEC cells. *Biochemical and Biophysical Research Communications* **207**(1):324-330.

Kennedy TG, Gillio-Meina C & Phang SH 2007 Prostaglandins and the initiation of blastocyst implantation and decidualization. *Reproduction* **134**:635–643.

Khalifeh-Soltani A, McKleroy W, Sakuma S, Cheung YY, Tharp K, Qiu Y, Turner SM, Chawla A, Stahl A & Atabai K 2014 Mfge8 promotes obesity by mediating the uptake of dietary fats and serum fatty acids. *Nature Medicine* **20**(2):175-183.

Kim H, Cruz M, Bourdeau A & Dumont DJ 2013 Cell-cell interactions influence vascular reprogramming by Prox1 during embryonic development. *PLoS One* **8**(1):e52197.

Kim J, Kang SG, Kim JI, Park JH, Kim SK, Cho DJ & Kim H 2006 Implication of ADAM-8, -9, -10, -12, -15, -17, and ADAMTS-1 in implantational remodeling of a mouse uterus. *Yonsei Medical Journal* **47**(4):558-567.

712 **Kolakowska J, Souchelnytskyi S, Saini RKR & Franczak A** 2017 Proteomic analysis of the
713 endometrium during early pregnancy in the domestic pig. Reproduction Fertility and
714 Development **29**(11):2255-2268.

715 **Kowalewski MP, Kautz E, Högger E, Hoffmann B & Boos A** 2014 Interplacental uterine
716 expression of genes involved in prostaglandin synthesis during canine pregnancy and at induced
717 prepartum luteolysis/abortion. *Reproductive Biology and Endocrinology* **12**:46.

718 **Kraeling RR, Rampacek GB & Fiorello NA** 1985 Inhibition of pregnancy with indomethacin
719 in mature gilts and prepuberal gilts induced to ovulate. *Biology of Reproduction* **32** 105–110.

720 **Kunau WH & Dommes P** 1978. Degradation of unsaturated fatty acids. Identification of
721 intermediates in the degradation of cis-4-decenoyl-CoA by extracts of beef-liver mitochondria.
722 *European Journal of Biochemistry* **91**:533–544.

723 **Lee KY, Jeong JW, Wang J, Ma L, Martin JF, Tsai SY, Lydon JP & DeMayo FJ** 2007
724 Bmp2 is critical for the murine uterine decidual response. *Molecular and Cellular Biology*
725 **27**:5468–5478.

726 **Marques E, Nkrumah JD, Sherman EL & Moore SS** 2009 Polymorphisms in positional
727 candidate genes on BTA14 and BTA26 affect carcass quality in beef cattle. *Journal of Animal*
728 *Science* **87**(8):2475-2484.

729 **McCracken JA, Custer EE & Lamsa JC** 1999 Luteolysis: a neuroendocrine-mediated event.
730 *Pysiological Reviews* **79**(2):263-323.

McKeehan WL, Sakagami Y, Hoshi H & McKeehan KA 1986 Two apparent human endothelial cell growth factors from human hepatoma cells are tumor-associated proteinase inhibitors. *Journal of Biological Chemistry* **261**(12):5378-83.

Morawska-Pucinska E, Szymanska M & Blitek A 2014 Expression profile and role of prostacyclin receptor (PTGIR) in peri-implantation porcine conceptuses. *Theriogenology* **82**(4):546-556.

Perry JS 1981 The mammalian fetal membranes. *Journal of Reproduction and Fertility* **62**:321–335.

Pierce KL, Fujino H, Srinivasan D & Regan JW 1999 Activation of FP prostanoid receptor isoforms leads to Rho-mediated changes in cell morphology and in the cell cytoskeleton. *Journal of Biological Chemistry* **274**(50):35944-35949.

Prins JR, Gomez-Lopez N & Robertson SA 2012 Interleukin-6 in pregnancy and gestational disorders. *Journal of Reproductive Immunology* **95**(1-2):1-14.

Ross JW, Ashworth MD, Stein DR, Couture OP, Tuggle CK & Geisert RD 2009 Identification of differential gene expression during porcine conceptus rapid trophoblastic elongation and attachment to uterine luminal epithelium. *Physiological Genomics* **36**:140-148.

Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M *et al.* 2003 TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* **34**:374–378.

Sales KJ, List T, Boddy SC, Williams AR, Anderson RA, Naor Z & Jabbour HN 2005 A novel angiogenic role for prostaglandin F2alpha-FP receptor interaction in human endometrial adenocarcinomas. *Cancer Research* **65**(17):7707-7716.

Seo H, Choi Y, Shim J, Yoo I & Ka H 2014 Prostaglandin transporters ABCC4 and SLCO2A1 in the uterine endometrium and conceptus during pregnancy in pigs. *Biology of Reproduction* **100**:1-10

Smyth GK 2004 Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Statistical Applications in Genetics and Molecular Biology* **3**:Article3.

Song G, Dunlap KA, Kim J, Bailey DW, Spencer TE, Burghardt RC, Wagner GF, Johnson GA & Bazer FW 2009 Stanniocalcin 1 is a luminal epithelial marker for implantation in pigs regulated by progesterone and estradiol. *Endocrinology* **150**(2):936-945.

Tan Y, AlKhamees B, Jia D, Li L, Couture JF, Figeys D, Jinushi M & Wang L 2015 MFG-E8 Is Critical for Embryonic Stem Cell-Mediated T Cell Immunomodulation. *Stem Cell Reports* **5**(5):741-752.

Taylor MR, Couto JR, Scallan CD, Ceriani RL & Peterson JA 1997 Lactadherin (formerly BA46), a membrane-associated glycoprotein expressed in human milk and breast carcinomas, promotes Arg-Gly-Asp (RGD)-dependent cell adhesion. *DNA Cell Biology* **16**(7):861-869.

Tinel H, Denker HW & Thie M 2000 Calcium influx in human uterine epithelial RL95–2 cells triggers adhesiveness for trophoblast-like cells: model studies on signalling events during embryo implantation. *Molecular Human Reproduction* **6**:1119–1130.

Tuo W, Harney JP & Bazer FW 1996 Developmentally regulated expression of interleukin-1 beta by peri-implantation conceptuses in swine. *Journal of Reproductive Immunology* **31**(3):185-198.

Ulbrich SE, Schulke K, Groebner AE, Reichenbach HD, Angioni C, Geisslinger G & Meyer HH 2009 Quantitative characterization of prostaglandins in the uterus of early pregnant cattle. *Reproduction* **138**(2):371-382.

Vilella F, Ramirez L, Berlanga O, Martínez S, Alamá P, Meseguer M, Pellicer A & Simón C 2013 PGE2 and PGF2α concentrations in human endometrial fluid as biomarkers for embryonic implantation. *Journal of Clinical Endocrinology and Metabolism* **98**(10):4123-4132.

Vonnahme KA, Fernando SC, Ross JW, Ashworth MD, DeSilva U, Malayer JR & Geisert RD 2004 Porcine endometrial expression of kininogen, factor XII, and plasma kallikrein in cyclic and pregnant gilts. *Biology of Reproduction* **70**(1):132-138.

Vonnahme KA, Malayer JR, Spivey HO, Ford SP, Clutter AC & Geisert RD 1999 Detection of kallikrein gene expression and enzymatic activity in porcine endometrium during the estrous cycle and early pregnancy. *Biology of Reproduction* **61**:1235–1241.

Waclawik A 2011 Novel insights into the mechanisms of pregnancy establishment: regulation of prostaglandin synthesis and signaling in the pig. *Reproduction* **142**:389–399.

Waclawik A & Ziecik AJ 2007 Differential expression of prostaglandin (PG) synthesis enzymes in conceptus during peri-implantation period and endometrial expression of carbonyl reductase/PG 9-ketoreductase in the pig. *Journal of Endocrinology* **194** 499-510.

Waclawik A, Kaczynski P & Jabbour HN 2013 Autocrine and paracrine mechanisms of prostaglandin E_2 action on trophoblast/conceptus cells through the prostaglandin E_2 receptor (PTGER2) during implantation. *Endocrinology* 154(10) 3864-3876.

Waclawik A, Rivero-Muller A, Blitek A, Kaczmarek MM, Brokken LJ, Watanabe K, Rahman NA & Ziecik AJ 2006 Molecular cloning and spatiotemporal expression of prostaglandin F synthase and microsomal prostaglandin E synthase-1 in porcine endometrium. *Endocrinology* **147** 210-221.

Waclawik A, Jabbour HN, Blitek A & Ziecik AJ 2009 Estradiol-17beta, prostaglandin E2 (PGE2), and the PGE2 receptor are involved in PGE2 positive feedback loop in the porcine endometrium. *Endocrinology* **150** 3823-3832.

Waclawik A, Kaczmarek MM, Kowalczyk AE, Bogacki M & Ziecik AJ 2008 Expression of prostaglandin synthesis pathway enzymes in the porcine corpus luteum during the oestrous cycle and early pregnancy. *Theriogenology* **70**(2):145-152.

Waclawik A, Kaczmarek MM, Blitek A, Kaczynski P & Ziecik AJ 2017 Embryo-maternal dialogue during pregnancy establishment and implantation in the pig. *Molecular Reproduction and Development* **84**(9):842-855.

Waite C, Mejia R & Ascoli M 2016 Gq/11-Dependent Changes in the Murine Ovarian Transcriptome at the End of Gestation. *Biology of Reproduction* **94**(3):62.

Wang CC, Yim KW, Poon TC, Choy KW, Chu CY, Lui WT, Lau TK, Rogers MS & Leung TN 2007 Innate immune response by ficolin binding in apoptotic placenta is associated with the clinical syndrome of preeclampsia. *Clinical Chemistry* **53**:42–52.

Wang Y, Zhao AM & Lin QD 2010 Role of cyclooxygenase-2 signaling pathway dysfunction in unexplained recurrent spontaneous abortion. *Chinese Medical Journal* **123**(12):1543-1547.

Watanabe T, Nakao A, Emerling D, Hashimoto Y, Tsukamoto K, Horie Y, Kinoshita M & Kurokawa K 1994 Prostaglandin F2 alpha enhances tyrosine phosphorylation and DNA synthesis through phospholipase C-coupled receptor via Ca(2+)-dependent intracellular pathway in NIH-3T3 cells. *Journal of Biological Chemistry* **269**(26):17619-17625.

Whitaker M 2006 Calcium at fertilization and in early development. *Physiological Reviews* **86**:25–88.

Zavy MT, Bazer FW, Thatcher WW & Wilcox CJ 1980 A study of prostaglandin F2α as the luteolysin in swine: V. Comparison of prostaglandin F, progestins, estrone and estradiol in uterine flushings from pregnant and nonpregnant gilts. *Prostaglandins* **20**:837–851.

Zhang J, Sun R, Wei H, Wu D & Tian Z 2007 Toll-like receptor 3 agonist enhances IFN-gamma and TNF-alpha production by murine uterine NK cells. *International Immunopharmacology* **7**(5):588-596.

Zhang S, Liu B, Gao L, Mao W, Fu C, Duritahala D, Zhang N, Zhang Y, Shen Y & Cao J 2017 Prostaglandin F2α-PTGFR signaling activation, growth factor expression and cell proliferation in bovine endometrial explants. *Reproduction Fertility and Development* **29**(11):2195-2205.

Zhao S & Fernald RD 2005 Comprehensive algorithm for quantitative real-time polymerase chain reaction. *Journal of Computational Biology* **12**(8):1047-1064.

833 **FIGURE LEGENDS**

834 **Figure 1.** Expression of PTGFR mRNA (A) and protein (B) in the porcine
835 conceptuses/trophoblasts at different stages of development. The representative samples of
836 Western blots are shown in the upper panels. Data are represented as the mean ± SEM. Bars with
837 different letter differ significantly ($p<0.05$).

838 **Figure 2.** Heatmap showing microarray analysis of DEGs in PGF2α-treated porcine trophoblast
839 cells (PGF2α 1-4), and corresponding vehicle-treated controls (Control 1-4). Normalized
840 expression data was clustered based on pair wise Pearson correlation using identified DEGs (red:
841 correlation=1; blue: correlation= -1). 576 genes were differentially expressed between the two
842 groups. A total of 360 genes were up-regulated and 216 genes were down-regulated ($p<0.05$).
843 The full list of DEGs is provided in Supplementary Table 1.

844 **Figure 3.** Functional map showing shared and treatment-specific functional annotation terms
845 generated in multi-cluster gene functional enrichment analysis for DEGs identified in porcine
846 primary trophoblast cells treated with PGF2α or vehicle (control). Analysis was performed in
847 ToppCluster software, functional map was edited and adjusted using Cytoscape. The redundant
848 and non-informative terms were removed. Complete results are presented in tabular format in
849 Supplementary Table 4.

850 **Figure 4.** Expression of selected target genes determined by quantitative (real-time) RT-PCR in
851 porcine primary trophoblast cells treated with PGF2α (100 nM, 1 μM) or vehicle (control). (A-D,
852 F-Y). *TIMP1*-metallopeptidase inhibitor 1, *TIMP2*-metallopeptidase inhibitor 2, *MMP2*-matrix
853 metallopeptidase 2, *MMP9*-matrix metallopeptidase 9, *BGN*-biglycan, *THBS1*-thrombospondin
854 1, *VTN*-vitronectin, *ADAM9*-ADAM metallopeptidase domain 9, *MFGE8*-milk fat globule-EGF

855 factor 8 protein, *PROX1*-prospero homeobox 1, *TGFB3*-transforming growth factor beta 3,
856 *LPAR1*-lysophosphatidic acid receptor 1, *AMBP*-alpha-1-microglobulin/bikunin precursor,
857 *BMP2*-bone morphogenetic protein 2, *IL1A*-interleukin 1 alpha, *IL6R*-interleukin 6 receptor,
858 *MASP1*-mannan binding lectin serine peptidase 1, *AGER*-advanced glycosylation end-product
859 specific receptor, *TLR3*-toll-like receptor 3, *STC2*-stanniocalcin 2, *S100G*-S100 calcium binding
860 protein G, *DECR1*-2,4-dienoyl-CoA reductase 1, *SLCO2A1*-solute carrier organic anion
861 transporter family member 2A1. Expression of *MMP9* mRNA in the porcine conceptuses/
862 trophoblasts at different stages of development (E). Data on panels A-D and F-Y are presented as
863 means ± SEM of fold change versus control (vehicle). Data on panel E is presented as mean ±
864 SEM. Bars with different letter differ significantly ($p<0.05$).

865 **Figure 5.** PGF2α increased adhesion to extracellular matrix protein - fibronectin (A) and
866 proliferation (B) of primary porcine trophoblast cells. Effect of PGF2α on MAPK1/3 (C) and
867 FAK (D) phosphorylation in porcine primary trophoblast cells *in vitro*. NCS-newborn calf serum
868 (used as a positive control), FLP – fluprostenol (stable prostaglandin F2α analogue), AL8810 –
869 PTGFR antagonist. Data are presented as means ± SEM of fold change versus control (vehicle).
870 Different letters (a-c) indicate statistically significant differences ($p<0.05$).

871 **SUPPLEMENTARY DATA LEGENDS**

872 **Supplementary Figure 1** Light microscopy with phase contrast (A) and immunofluorescent
873 staining for cytokeratin presence (B) in trophoblast cells isolated from day 15 conceptuses.
874 Negative control (mouse IgG) is shown in lower-left part of the figure. Arrows indicate signals
875 for cytokeratin.

876 **Supplementary Figure 2.** Gene expression distance matrix of analyzed samples generated in
877 Geneplotter library. VSN-normalized microarray expression data was clustered based on pair
878 wise correlation using all detectable probes. PGF2α_1-4 – porcine trophoblast cells treated with
879 PGF2α (1 μM), isolated from gilts 1-4. Control_1-4 – porcine trophoblast cells treated with
880 vehicle (0.01% ethanol) isolated from gilts 1-4.

881 **Supplementary Figure 3.** Selected functional terms overrepresented by DEGs identified by IPA
882 analysis determined basing on the identified canonical pathways. Complete results from IPA
883 analyses are presented in tabular format in Supplementary Tables 5-7.

884 **Supplementary Table 1.** Results of statistical analysis of microarray data (paired LIMMA).

885 **Supplementary Table 2.** DAVID functional annotation clustering results for up-regulated genes
886 in PGF2α-treated conceptus cells compared to vehicle-treated conceptus cells.

887 **Supplementary Table 3**. DAVID functional annotation clustering results for down-regulated
888 genes in PGF2α-treated conceptus cells compared to vehicle-treated conceptus cells.

889 **Supplementary Table 4.** Enriched GO terms and pathways by up-regulated genes in PGF2α-
890 treated conceptus cells compared to vehicle-treated conceptus cells.

891 **Supplementary Table 5.** Canonical pathways potentially regulated by identified DEGs.

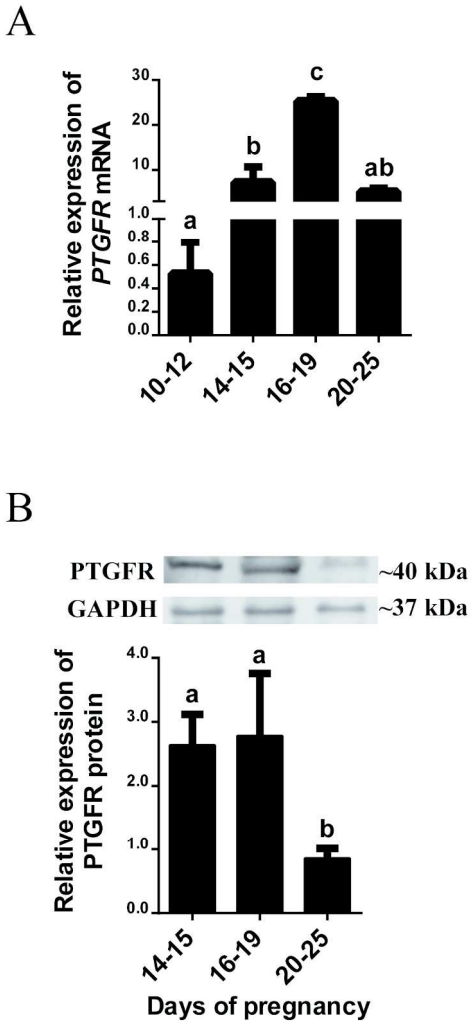
892 **Supplementary Table 6.** Functions affected by identified DEGs.

893 **Supplementary Table 7** Upstream regulators affecting expression of identified DEGs.

894 **Supplementary Table 8.** Comparison of results from qPCR and microarray gene expression
895 analyses.

896

FIGURE 1



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117x271mm (600 x 600 DPI)

FIGURE 2

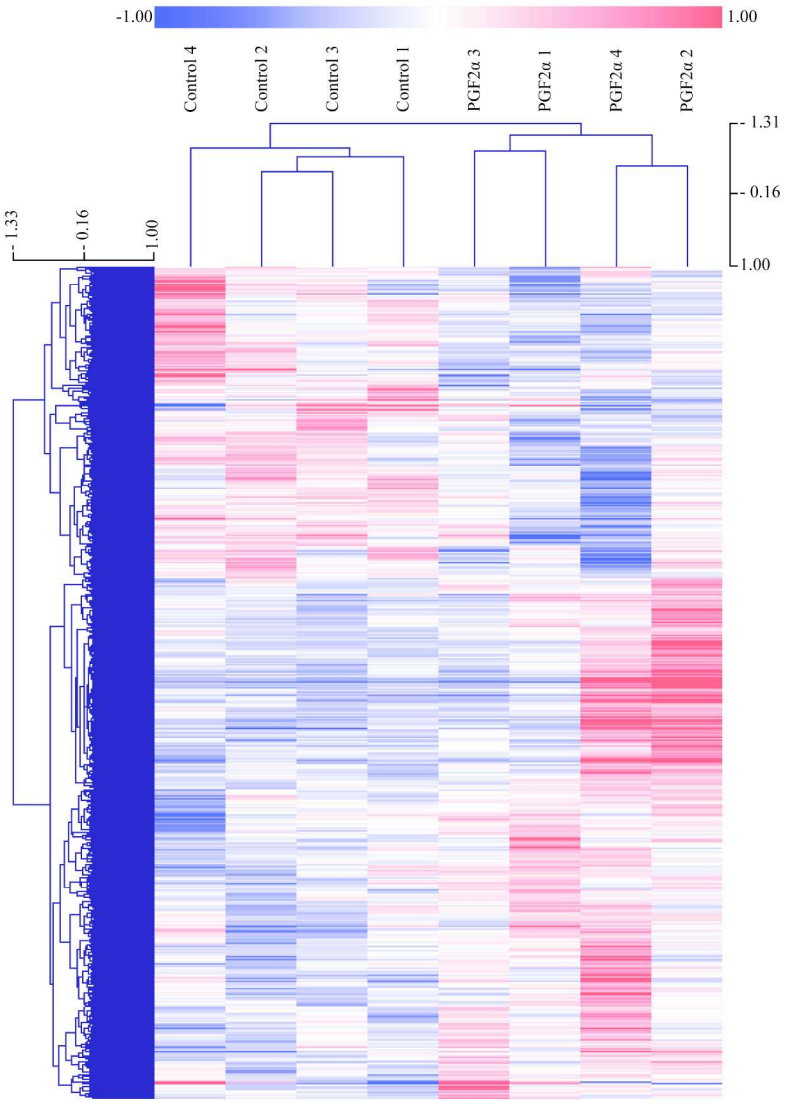

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FIGURE 3

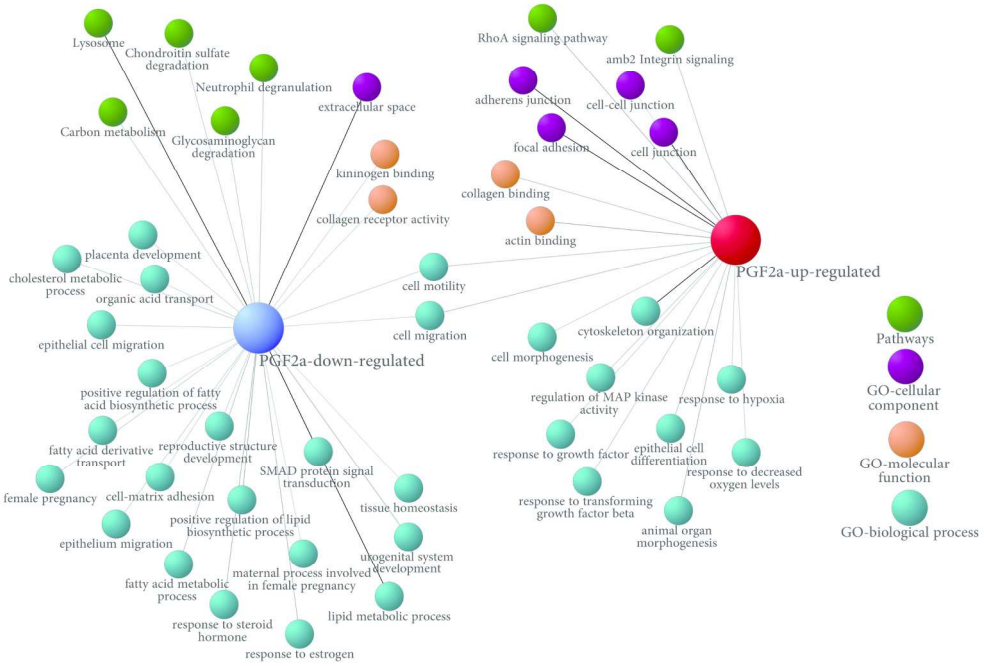

Figure 3. Functional map showing shared and treatment-specific functional annotation terms generated in multi-cluster gene functional enrichment analysis for DEGs identified in porcine primary trophoblast cells treated with PGF2α or vehicle (control). Analysis was performed in ToppCluster software, functional map was edited and adjusted using Cytoscape. The redundant and non-informative terms were removed. Complete results are presented in tabular format in Supplementary Table 8.

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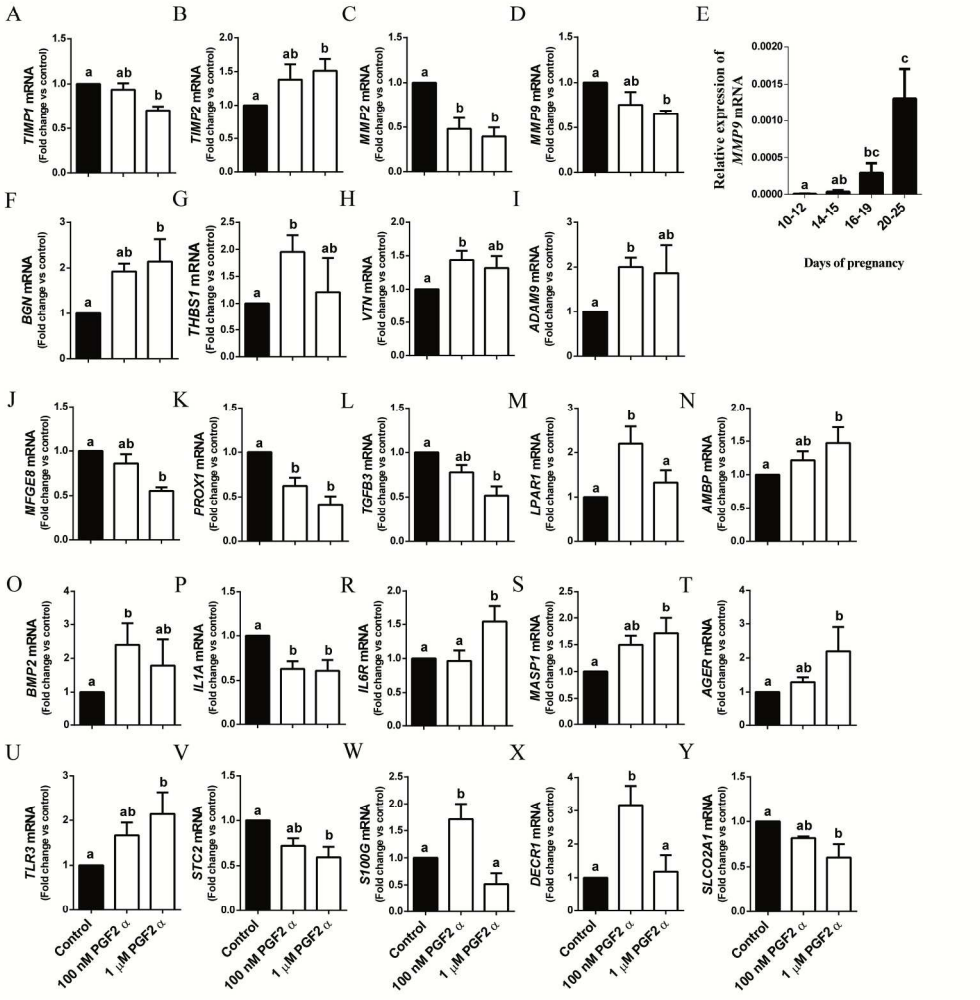
FIGURE 4



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205x224mm (300 x 300 DPI)

FIGURE 5

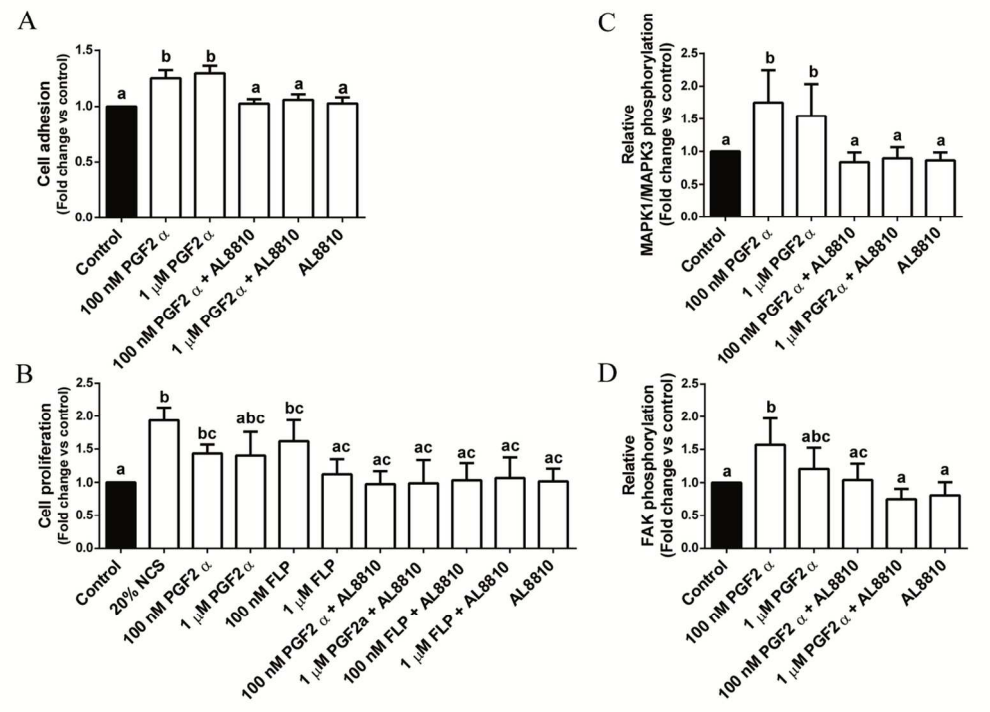

Figure 5. PGF2α increased adhesion to extracellular matrix protein - fibronectin (A) and proliferation (B) of primary porcine trophoblast cells. Effect of PGF2α on MAPK1/3 (C) and FAK (D) phosphorylation in porcine primary trophoblast cells in vitro. NCS-newborn calf serum (used as a positive control), FLP – fluprostenol (stable prostaglandin F2α analogue), AL8810 – PTGFR antagonist. Data are presented as means ± SEM of fold change versus control (vehicle). Different letters (a-c) indicate statistically significant differences ($p<0.05$).

121x97mm (300 x 300 DPI)

Table 1. Primer sequences and assays used in qPCR analyses.

Gene	Primer Sequence / TaqMan® Assay ID	RefSeq ID / GenBank Accession No.	Reference
PTGFR	sense: 5'-TCAGCAGCACAGACAAGG-3 antisense: 5'-TTCACAGGCATCCAGATAATC-3	NM_214059	Kaczynski & Waclawik 2013
ACTB	Sense: 5'-ACATCAAGGAGAAGCTCTGCTACG-3' Antisense: 5'-GAGGGGCGATGATCTTGATCTTCA-3'	U07786	Kaczynski & Waclawik 2013
GAPDH	Sense: 5'-CAGCAATGCCTCCTGTACCA-3' Antisense: 5-GATGCCGAAGTTGTCATGGA-3'	AF017079	Kaczynski & Waclawik 2013
PPIA	Sense: 5'-TAACCCCACCGTCTTCTT-3' Antisense: 5'-TGCCATCCAACCACTCAG-3'	AY266299.1	Kaczynski & Waclawik 2013
ADAM9	Ss03373282_m1	AF069646.1	-
AGER	Ss03390846_g1	EU282357.1	-
AMBP	Ss03374771_m1	AK232748.1	-
BGN	Ss03375454_u1	AF159382.1	Kaczynski *et al*. 2016
BMP2	Ss03373798_g1	AY669080	-
DECR1	Ss01051816_m1	AK230726.1	-
IL1A	Ss03391335_m1	X52731.1	Kaczynski *et al*. 2016
IL6	Ss03384604_u1	AF309651.1	Kaczynski *et al*. 2016
KNG1	Ss03373690_m1	AY321363.1	-
LPAR1	Ss03377225_u1	AK344044.1	-
MASP1	Ss04246770_m1	GU810082.1	-
MFGE8	Ss03390111_m1	AK233926.1	-
MMP2	Ss03394318_m1	AF295805.1	-
MMP9	Ss03392100_m1	DQ132879.1	Kaczynski *et al*. 2016
PROX1	Ss04246028_m1	EF486324.1	-
S100G	Ss03382848_u1	L13068.1	-
SLCO2A1	Ss03390713_m1	NM_001123195	-
STC2	Ss03389624_m1	AK349078.1	-
TGFB3	Ss03394352_m1	X14150.1	Kaczynski *et al*. 2016
THBS1	Ss03373620_m1	AM238691.1	-
TLR3	Ss03388861_m1	AB111939.1	-
VTN	Ss03382603_u1	AK232304.1	-
ACTB	Ss03376081_u1	AK237086.1	Kaczynski *et al*. 2016
PPIA	Ss03394782_g1	AY266299.1	Kaczynski *et al*. 2016
GAPDH	Ss03375435_u1	NM_001206359.1	Kaczynski *et al*. 2016

Table 2. List of antibodies used in Western Blot analyses and immunolocalization experiments.

Peptide/Protein Target	Antigen Sequence	Name of Antibody	Manufacturer, Catalog No., or Name of Source	Species Raised in Monoclonal or Polyclonal	Dilution used
PTGFR	QRFRQKSKASFLLLASGLVITDFFGHLI NGAIAVFVYASDKEWIRFDQSNVLCSI	PGF2aR antibody (H-55)	Santa Cruz Biotechnology, sc-67029	Rabbit, polyclonal	1:50
MAPK1/3	N/A	p44/42 MAPK (Erk1/2) antibody	Cell Signaling, #9102S	Rabbit, polyclonal	1:300
phospho-MAPK1/3	N/A	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody	Cell Signaling, #9101	Rabbit, polyclonal	1:300
FAK	N/A	Focal Adhesion Kinase (FAK) antibody	Cell Signaling, #3285S	Rabbit, polyclonal	1:300
phospho-FAK	N/A	Phospho-FAK (Tyr397) antibody	Cell Signaling, #3283	Rabbit, polyclonal	1:300
Cytokeratin	N/A	Anti-Pan Cytokeratin antibody	Sigma-Aldrich C 9687	Mouse monoclonal	1:50
anti-rabbit HRP	N/A	Immun-Star™ Goat Anti-Rabit (GAR)-HRP Conjugate	Bio-Rad Laboratories, #170-5046	Goat, polyclonal	1:20000
anti-mouse	N/A	Cy™3 AffiniPure Donkey Anti-Mouse IgG	Jakcson ImmunoResearch # 715-165-150	Donkey, polyclonal	1:1000

Table 3. Selected results of DAVID functional annotation clustering for differentially expressed genes in trophoblast cells treated with PGF2*α*.

Selected functional terms of overrepresented annotation clusters	Enrichment score
UP REGULATED	
focal adhesion (30; 3.67); cell adhesion (50; 1.48); protein binding involved in cell adhesion (14; 2.29)	3.96
cell migration (42; 1.79); regulation of cell motility (28; 1.91)	2.91
response to hypoxia (17; 3.01)	2.84
response to fibroblast growth factor (10; 3.71); fibroblast growth factor receptor signaling pathway (8; 4.01)	2.53
response to growth factor (28; 2.23); response to transforming growth factor beta (13; 3.05); transmembrane receptor protein serine/threonine kinase signaling pathway (14; 2.19)	2.43
regulation of cell proliferation (19; 1.50)	2.32
response to steroid hormone (16; 2.11)	2.26
anatomical structure formation involved in morphogenesis (38; 1.67); angiogenesis (17; 2.07)	2.01
regulation of MAP kinase activity (18; 2.78); protein phosphorylation (50; 1.36)	1.95
regulation of epithelial cell proliferation (14; 2.35)	1.68
organ morphogenesis (36; 1.83); epithelium development (32; 1.52)	1.52
extracellular matrix binding (5; 4.72)	1.35
DOWN REGULATED	
fatty acid biosynthetic process (6; 3.38)	2.71
regulation of intracellular signal transduction (34; 1.62)	2.04
steroid biosynthetic process (7; 3.91)	1.89
cell migration (28; 1.93)	1.85
anion transport (14; 2.44)	1.79
regulation of cell proliferation (31; 1.63)	1.78
extracellular matrix organization (11; 2.69)	1.74
female pregnancy (8; 3.49)	1.65
cellular response to interferon gamma (6; 3.63)	1.34
placenta development (6; 3.38); reproductive structure development (11; 2.15)	1.35